

## **REMARKS**

The disclosure is objected to because of informalities. A Substitute Specification (a clean version and a marked-up version) reflects the appropriate corrections to overcome the objections. Withdrawal of the objection is respectfully requested.

Claim 20 is objected to because of an informality. The claim is amended to obviate the objection. Withdrawal of the objection is respectfully requested.

Claim 20 is rejected under 35 USC 112, second paragraph, for allegedly failing to particularly point out and distinctly claim the subject matter of the invention. The claim is amended to obviate the rejection. Withdrawal of the rejection is respectfully requested.

The Examiner correctly presumes that the subject matter of the various claims was commonly owned at the time the inventions covered in the application were made.

Claim 20 is rejected under 35 U.S.C. 103(a) as unpatentable over Palmer et al. ("Vitellogenin as a Biomarker for Xenobiotic Estrogens in an Amphibian Model System") in view of Dunbar et al. ("Preparation of Polyclonal Antibodies"). The rejection is respectfully traversed.

As specified in the amended claim 20, the present invention is characterized in that purifying IgG by performing an adsorption purification using a column coupled with blood serum proteins of a male frog and an affinity purification using a column coupled with a frog vitellogenin and eventually obtaining polyclonal antibody specific to a frog vitellogenin. By this method, a polyclonal antibody specific to a frog vitellogenin with high degree of specificity can be obtained.

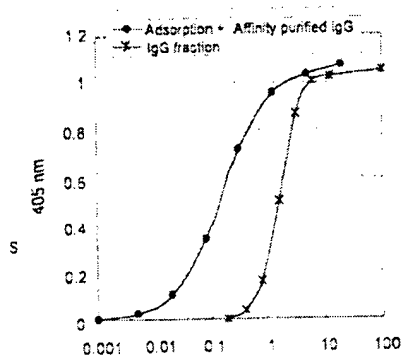
In other words, antibody against the protein in the normal male frog is eliminated by an adsorption purification using a column coupled with blood serum proteins of a male frog and an immune reaction with a normal male blood serum proteins would not

occur any more, thus polyclonal antibody specific to a frog vitellogenin with high degree of specificity can be obtained. At this point, there is a possibility that a minute amount of protein common to male and female other than vitellogenin being incorporated in antigen to be immunized, and, if it being the case, an antibody against proteins other than vitellogenin may develop. Especially, when the contaminant has high degree of antigenicity, a consistent formation of antibody is to be induced even the amount of incorporated material is very small. According to the present invention, unwanted antibodies can be eliminated by adsorption purification with normal male blood serum proteins containing no vitellogenin. In addition, polyclonal antibody specific to a frog vitellogenin is a mixture of antibodies that recognizes various sites of vitellogenin protein and when there is commonly recognized antigen site, such as sugar chain and phosphorylated amino acid, among various kinds of proteins of the male blood serum, protein other than vitellogenin is also recognized which would be the cause of nonspecific reaction. According to the present invention, antibody that has cross-reactivity can positively be eliminated by adsorption purification.

Furthermore, affinity purification with vitellogenin antigen enables to selectively collect anti-vitellogenin antibody, thus specificity of polyclonal antibody specific to a frog vitellogenin can be increased.

The present invention increases the specificity of obtained polyclonal antibody specific to a frog vitellogenin to a great degree by performing both adsorption purification and affinity purification as described, compared to a case where purification is performed independently. In addition, in the ELISA method using polyclonal antibody specific to a frog vitellogenin obtained according to the present invention, native vitellogenin can specifically be detected even where various kinds of assay such as blood serum and blood may be used, and high sensitive detection without false positive reaction against normal male blood serum can be realized. In Palmer, antiserum is used in ELISA method as it is, without applying IgG fraction or purification, sensitivity of detecting vitellogenin is lower detection limit of about 10 to 20 ng/ml. To the contrary, in ELISA method using polyclonal antibody specific to a frog vitellogenin obtained

according to the present invention, high detection sensitivity of lower detection limit of 0.2 to 2 ng/ml can be realized. In addition, as shown in Fig. 1 below, when antibody being fractionated according to IgG and antibody purified by affinity purification after adsorption purification are compared, the latter has antibody value ten times higher than the former thus the antibody non-specific to vitellogenin is being eliminated effectively. With this method, sensitivity in ELISA method can be increased.



Antibody concentration (pg/ml)

Fig 1

Furthermore, antigen used in immunization is, for example, fractionated by column chromatography from blood serum of a male frog in which vitellogenin synthesis is induced. However, the obtained antigen may contain protein that is common to male and female other than vitellogenin. In such case, the antibody common to both male and female protein other than vitellogenin can be eliminated assuredly by performing both adsorption purification and affinity purification as in the present invention and the specificity and affinity of polyclonal antibody to a frog vitellogenin can be increased.

The present invention in the amended claims is compared with the cited references hereunder.

None of the references cited against the present invention describes performing BOTH the adsorption purification using a column coupled with blood serum proteins of a male frog and the affinity purification using a column coupled with a frog vitellogenin.

Palmer describes using serum in ELISA method and there is a passage about production of serum in a section titled "polyclonal antibody production". In the section titled "polyclonal antibody" describes obtaining serum from blood collected from a rabbit immunized using vitellogenin, however, there is no mention of IgG fraction nor of purification.

Dunbar describes obtaining IgG from serum taken from blood collected from a rabbit and purifying thereof. Three types of purification, namely, ammonium sulfate fractionation, DEAE anion exchange chromatography and affinity purification using protein A is described as a method of purifying IgG, however, there is no mention on an adsorption purification using a column coupled with blood serum proteins of a male frog and an affinity purification using a column coupled with a frog vitellogenin as in the present invention. Furthermore, although production of polyclonal antibody is described, there is no mention on the production of polyclonal antibody specific to a frog vitellogenin.

Kawahara describes obtaining sera from blood collected from an immunized rabbit and states "the prepared antisera were absorbed with normal male sera in order to obtain vitellogenin specific antisera". Nevertheless, there is no mention on an adsorption purification using a column coupled with blood serum proteins of a male frog or an affinity purification using a column coupled with a frog vitellogenin.

USP 5,153,117 describes a method using protein G and a method using chromatography by DEAE SEPHADEX® column as IgG purification method in order to fractionate labeled antibody IgG and unbound label. However, the reference does NOT state performing BOTH the adsorption purification using a column coupled with blood

serum proteins of a male frog and the affinity purification using a column coupled with a frog vitellogenin.

USP 5,304,496 states IgG fractionating blood serum from blood collected from immunized rabbit and purification. USP 5,304,496 mentions that the method of purification is as in described in Garvey and also using column coupled with antibody in the purification of antigen. Garvey describes affinity purification using column coupled with antigen as a general method, however, there is no description as to adsorption purification by bonding nonspecific antibody. Furthermore, the reference does NOT state performing BOTH the adsorption purification using a column coupled with blood serum proteins of a male frog and the affinity purification using a column coupled with a frog vitellogenin.

Applicant now particularly address the arguments of the Examiner as follows:

Palmer and Dunbar describe the production of polyclonal antibody. Palmer states production of polyclonal antibody specific to a frog vitellogenin, however, there is no mention on polyclonal antibody specific to a frog vitellogenin in Dunbar. As stated by the Examiner, there is no description on purification in Palmer while Dunbar mentions affinity purification by ammonium sulfate fractionation, DEAE anion exchange chromatography and Protein A. However, none of the two references describes performing BOTH the adsorption purification using a column coupled with blood serum proteins of a male frog and the affinity purification using a column coupled with a frog vitellogenin. The affinity purification described in Dunbar is used for purifying IgG, therefore, it does NOT have the same meaning as affinity purification of the present invention that is specific to the antigen and it is not related to specificity of antigen. For this reason, the effect of increasing specificity of polyclonal antibody specific to a frog vitellogenin by performing both the adsorption purification using a column coupled with blood serum proteins of a male frog and the affinity purification using a column coupled with a frog vitellogenin as described above CANNOT be obtained even the two

references are combined. Therefore, structure, function and effect of the present invention cannot be obtained even the two references are combined.

Kawahara describes adsorption purification using serum of a male frog containing protein, however, there is NO description on “adsorption purification using a column coupled with blood serum proteins of a male frog” or “affinity purification using a column coupled with a frog vitellogenin.” To the contrary, the present invention has an effect of increasing the effectiveness of adsorption purification by using a column coupled with blood serum proteins of a male frog. For this reason, function and effect of the present invention cannot be obtained by combining this reference with any of the other references.

USP 5,153,117 describes a method using protein G and a using chromatography of DEAE SEPHADEX® column. However, the method is to fractionate the labeled antibody from the unbound label and the method is NOT for producing antibody that is specific to the antigen. Therefore, the object of purifying IgG is different from the start, and combining references that belong to different technical fields as such is NOT obvious. The method using protein G is purification for purifying IgG, therefore, it does NOT have the same meaning as affinity purification of the present invention that is specific to the antigen and it is not related to specificity of antigen. For this reason, effect of increasing specificity of polyclonal antibody specific to a frog vitellogenin by performing both the adsorption purification using a column coupled with blood serum proteins of a male frog and the affinity purification using a column coupled with a frog vitellogenin as described above CANNOT be obtained even Palmer and USP 5,153,117 are combined. Therefore, structure, function and effect of the present invention cannot be obtained even this reference is combined with any of the other references.

Howevei as mentioned above, USP 5,304,496 does NOT describe the production of polyclonal antibody specific to a frog vitellogenin nor performing the adsorption purification using a column coupled with blood serum proteins of a male frog and the

affinity purification using a column coupled with a frog vitellogenin. When the affinity purification using column coupled with antigen as stated in Garvey, a reference cited in the cited reference, and Palmer is combined, though the antibody coupled with vitellogenin can be obtained, the antibody that shows cross reactivity against the protein of blood serum common to male and female cannot be eliminated, therefore, the increase in specificity of polyclonal antibody specific to a frog vitellogenin by performing BOTH the adsorption purification using a column coupled with blood serum proteins of a male frog and the affinity purification using a column coupled with a frog vitellogenin cannot be obtained. Therefore, structure, function and effect of the present invention cannot be obtained even this reference is combined with any of the other references.

Traditionally vitellogenin had been easily detected since the amount induced by natural estrogens thereof was immensely large and there had been no need for detecting vitellogenin in extremely high degree of sensitivity. For this reason, it is considered that the poorly purified antibody was able to give satisfactory results. However, use of such antibody was unsatisfactory for assessment of estrogenic effect of chemical substances on vitellogenin induction, because of its low sensitivity in detection. It is obligatory to prepare the highly purified antibody for such assessment. For this reason, it is effective to perform BOTH the adsorption purification for eliminating false positive reaction against normal male blood serum of a male frog and the affinity purification for highly sensitive detection. Since vitellogenin is a highly acidic and huge blood serum protein, there is a possibility of vitellogenin complexing with other blood proteins, forming a high order structure. If this is the case, there is a possibility that the structure hides some epitopes from recognition by the non-purified vitellogenin antibody. In addition, combination of proteins in the complex may variously change according to the status of the sample, for example, dissociation of the complex that may be caused by diluting the serum with some buffer solutions. Namely, in a case where the amount of native vitellogenin is detected and the quantity being determined by Sandwich ELISA, there is a risk that antibodies against non-vitellogenic antigen (i.e. antigen bound to vitellogenin) may also participate in detection of vitellogenin. In addition, vitellogenin

antibody molecules specific to the epitopes hidden in the complex, if present in the antibody preparation, may change estimation of vitellogenin amount according to the sample status. Moreover, the antibody molecules with low affinity to vitellogenin cannot work as a result of dilution of sample blood serum, possibly leading to a change in shape of quantification curve. For these reasons, the thorough and complete purification of the antibody is preferable for highly sensitive and quantitative method. The antibody appropriate for detecting vitellogenin in high sensitivity can be prepared by performing BOTH of the adsorption purification using a column coupled with blood serum proteins of a male frog and the affinity purification using a column coupled with a frog vitellogenin as in the present invention.

For the reasons explained above, it is respectfully submitted that claim 20 is allowable over the applied art.

Withdrawal of the rejection is respectfully requested.

Further, Applicants assert that there are also reasons other than those set forth above why the pending claims are patentable. Applicants hereby reserve the right to submit those other reasons and to argue for the patentability of claims not explicitly addressed herein in future papers.

In view of the foregoing, reconsideration of the application and allowance of the pending claims are respectfully requested. Should the Examiner believe anything further is desirable in order to place the application in even better condition for allowance, the Examiner is invited to contact Applicants' representative at the telephone number listed below.



Should additional fees be necessary in connection with the filing of this paper or if a Petition for Extension of Time is required for timely acceptance of the same, the Commissioner is hereby authorized to charge Deposit Account No. 18-0013 for any such fees and Applicant(s) hereby petition for such extension of time.

Dated: September 21, 2006

Respectfully submitted,

By 

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Attorney for Applicant

Enclosures: Amendment Transmittal  
Marked-Up Version of a Substitute Specification  
Clean Version of the Substitute Specification